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9-26-01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: **Hans Herweijer, Jon A. Wolff, Larry F. Whitesell and Matthew R. Wolff**

Serial No.: 09/330,909

Filed: 6/11/99

Group Art Unit: 1632

Examiner: **J. Woitach**

OIPE
SEP 14 2001
PATENT & TRADEMARK OFFICE

For: **A Process For Delivering Nucleic Acids To Cardiac Tissue**

DECLARATION UNDER 37 C.F.R. §1.131

Assistant Commissioner for Patents
Washington, DC 20231

(Considered
JW 11/26/01

Dear Sir:

I, Hans Herweijer, hereby declare as follows:

1. I am an inventor of the captioned application.

2. Applicants' process was conceived prior to the publication date of the Office Action prior art reference to Hajjar *et al.*

3. We developed our recharging process with due diligence from conception to the filing of our application.

4. Photocopies of laboratory notebook pages dated March and April, 1998 accompany this Declaration for support.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Herweijer 9/12/01

Hans Herweijer | Date

Naked plasmid DNA into pig coronary arteries - experiment 2

980331 injection

Bed 1 (circumflex) – injection into artery
Block both artery and vein
Inject 6 ml papaverine (1 mg), wait 5 min
Release block for 30 seconds
Inject 25 ml pCI-Luc+ (1250 µg) in 22 seconds, wait 2 minutes before releasing block; at start of injection there was an immediate arrest, requiring two shocks; the injection area turned light, slowly returning to pink/red, faster after release blocks

Bed 2 (tissue)
Inject 500 µl (500 µg) pCI-Luc+ directly

SAMPLE	RLU
1	287
2	39716
3	202310
4	144085

980402 extract heart

			SAMPLE	RLU
Bed 1	1.	circumflex, endo, lateral	5	1312
	2.	circumflex, endo, lateral	6	217019
	3.	circumflex, endo, lateral	7	2768950
	4.	circumflex, endo, medial	8	291736
	5.	circumflex, endo, medial	9	36279
	6.	circumflex, endo, medial	10	473012
	7.	circumflex, epi, lateral	11	42433
	8.	circumflex, epi, lateral	12	68436
	9.	circumflex, epi, lateral	13	625494
	10.	circumflex, epi, medial	14	46659
	11.	circumflex, epi, medial	15	14010
	12.	circumflex, epi, medial	16	127449
Bed 2	13.	circumflex, endo, lateral	17	292241
	14.	circumflex, endo, medial	18	36212
	15.	circumflex, epi, lateral	19	13276
	16.	circumflex, epi, medial	20	3322
Bed 3	17.	control, posterior, endo, lateral	21	942
	18.	control, posterior, endo, media	22	1328
	19.	control, posterior, epi, lateral	23	1329
	20.	control, posterior, epi, medial	24	679

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Dear Sir:

I, Hans Herweijer, hereby declare as follows:

1. I am an inventor of the captioned application.

2. The inventors submit with this Declaration and Response further experimental material (attached) illustrating *in vivo* delivery and expression of nucleic acids yielding therapeutic proteins in pigs.

3. The material is consistent with the specification as filed and only methods described in the specification have been used. No new matter was used in the experiments.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Herweijer 9/7/01

Hans Herweijer Date

Increased coronary blood flow following VEGF gene therapy

A porcine chronic coronary occlusion model was used to determine whether percutaneous cardiac intravenous injection of a vascular endothelial growth factor (VEGF) expression vector improves regional myocardial blood flow (both at rest and during tachycardia) and whether this gene therapy procedure ameliorates inducible ischemia. Chronic coronary occlusion is achieved in "mini-pigs" by placement of an ameroid constrictor around the upper great cardiac artery.

Porcine model of chronic coronary occlusion
Yucatan mini-pigs (20-30 kg) were sedated with telezol (20-30mg IM), induced with thiopental sodium (250-500 mg IV) and endotracheally intubated. Anesthesia was maintained with inhaled isoflurane (0.5 - 3%). The heart was exposed via a left lateral thoracotomy, and an ameroid constrictor (Research Instruments and MFG, Corvallis, OR) was placed around the upper great cardiac artery. Endocardial and epicardial ultrasonographic crystals (Sonometrics, Inc., London, Ontario) were sutured in place to provide heart-wall thickness measurements. Right atrial pacing wires and left atrial and arterial cannula were tunneled subcutaneously and externalized at the base of the animal's neck. The thoracotomy was closed in layers, and the animal allowed to recover. Cefazolin (1 g IV) was used as a perioperative antibiotic, and banamine (25 mg IM) was administered immediately after surgery and daily for 3 days for analgesia.

One to two days after constrictor placement, heart wall thickness and myocardial blood flow was determined (using microspheres) at rest and tachycardia. Tachycardia was induced using the pacing wires, or by dobutamine treatment. These measurements provided base line data for each animal. The measurements were repeated after 3 weeks (at which time great cardiac artery blood flow should be blocked). One to two days later, gene therapy was performed (see below). All measurements were repeated 3 weeks later, at which time a possible effect of neovascularization could be observed. Following sacrifice, heart tissue was examined by histology.

Percutaneous injection of pDNA (pCI-VEGF or pCI-Luc) into cardiac veins
Gene transfer was performed by infusion of plasmid DNA solutions into the great cardiac vein. The right carotid artery was exposed via a midline incision and cannulated with an 8-9 Fr introducer sheath. A 10.5-11 Fr sheath was placed in the right jugular vein. The left main coronary artery was engaged with a coronary angiographic catheter and a left coronary artery angiogram was obtained. A modified 10 Fr guiding catheter was advanced through the jugular sheath into the coronary sinus. The delivery catheter (modified triple-lumen 7 Fr balloon-tipped pulmonary artery catheter with an infusion lumen, a balloon inflation/deflation lumen and an end-hole lumen for intravascular pressure measurements) was advanced over a guidewire into the proximal great (anterior) cardiac vein. The vein was occluded by inflating the latex balloon on the distal tip of the catheter. Low pressure injections of diluted iodinated contrast were used, in conjunction with the left coronary angiogram, to delineate the myocardial territory drained by the vein. Retrograde infusions of 5-30 ml of pDNA solution were performed using a modified cath lab injection pump (Acist CL100 Injection System, Acist Medical, Eden

low pressure

Prairie, MN). This injection pump is controlled by a pressure transducer, such that a constant, preset, injection pressure is maintained until the desired volume is delivered. Pressure measurements were done via a small fluid-filled catheter threaded inside the large injection catheter (measuring pressure at the tip of the injection catheter). A more ideal pressure measurement system would use pressure transducing guidewires, as they would take up less of the injector catheter lumen. While we provided the pressure-to-injection pump bio-feedback loop in a separate computer (Apple PowerBook, programmed in LabView, National Instruments, Austin, TX), this could easily be incorporated into the Acist injection system. With this constant pressure system, pDNA is delivered as efficiently as with constant flow rate injection, yet with the advantage of added safety.

The occlusion balloon was left inflated for 2 minutes following the injection. Cardiac venous and aortic pressures and a limb-lead electrocardiogram were recorded during the retrograde venous injections. Following gene delivery, both vessels were ligated and the neck incision was closed.

Rapid-pacing stress testing. Regional systolic myocardial wall thickening will be measured by two methods at resting heart rates and during atrial pacing at 150 and 200 beats/min. These studies were performed on concious, unsedated pigs resting comfortably in a sling. The degree of systolic posterolateral wall thickening (fractional thickening or systolic thickness/diastolic thickness) was determined using the chronically implanted ultrasound crystals, and recorded, analyzed and stored on a personal computer using a dedicated hardware and software system (Sonometrics, Inc., London, Ontario). Myocardial ischemia was quantitated as % difference in fractional wall thickening between baseline and rapid pacing measurements.

The extent of myocardial ischemia during rapid pacing was assessed by two-dimensional trans-thoracic echocardiography (2 dimensional trans-thoracic parasternal short axis views obtained at resting heart rates and during atrial pacing at 150 and 200 beats/min using a 3.5 MHz transducer, model number 77065AC, Hewlett-Packard, Andover, MA). Extent of myocardial ischemia was quantitated as the fractional thickening (systolic wall thickness/diastolic wall thickness) determined in each of 12 equal 30-degree radial segments. The extent of ischemia was defined as the number of segments with a 25% or greater decline in fractional thickening with rapid pacing, relative to baseline images.

Regional myocardial blood flow. Regional myocardial blood flow was assessed in conscious, unsedated pigs both at resting heart rates and during rapid atrial pacing (200 beats/min) using fluorescent microspheres (NuFlow, Triton Technology, San Diego, CA). Approximately 2 x 10^6 15-nm microspheres labeled with one of eight available different fluorescent dyes, suspended in 10% dextran mixed with Tween detergent, were injected into the left atrial catheter as blood is simultaneously withdrawn from the arterial catheter at a constant flow rate. Microsphere blood flow determinations were made with different dyes at resting heart rates and with atrial pacing at 150 and 200 beats/min. Following terminal studies, the target bed is cut into ~1†cm^3 sections, and a corresponding number of sections are obtained from the non-ischemic bed. Each section is further divided into

subendocardial and subepicardial halfs, the subsequent sections weighed, and the number of retained microspheres are counted by flow cytometry (Triton Technology). Raw counts are corrected for background and crossover, and compared to the reference arterial sample to provide regional flow in units of milliliters per minute per gram myocardium. Endocardial, epicardial and the ratio of endocardial to epicardial blood flow are calculated as averages for the ischemic and non-ischemic regions at each heart rate.

Serum markers of myocardial injury. Serum creatine phosphokinase activity was determined from blood aspirated from the left atrial catheter (assay performed at UW Hospital and Clinics clinical laboratory).

RESULTS

Ischemia was induced in a pig by placement of an ameroid constrictor around the great cardiac artery. At baseline (2 days following placement of the constrictor, at which time point blood flow should remain unrestricted), blood flow increases in all samples during stress (baseline stress), relative to rest (baseline rest), see Figure. No stress-induced increases were measured in the LAD (great cardiac vein) region and the left ventricle 3 weeks after placement of the constrictor (ischemic rest v. ischemic stress). Gene transfer of 9.95 mg pCI-VEGF (in which human vascular endothelial growth factor 165 is under transcriptional control of the human cytomegalovirus promoter) followed at this time point. Three weeks later, blood flow measurements showed a highly significant increase in blood flow in the LAD (great cardiac vein) region and the left ventricle upon stress (post therapy stress). Increased blood flow at this time point was limited to the epicardium, consistent with the region where gene delivery took place. Note that the right ventricular region is not affected, as the ameroid constrictor does not limit blood flow to this area.

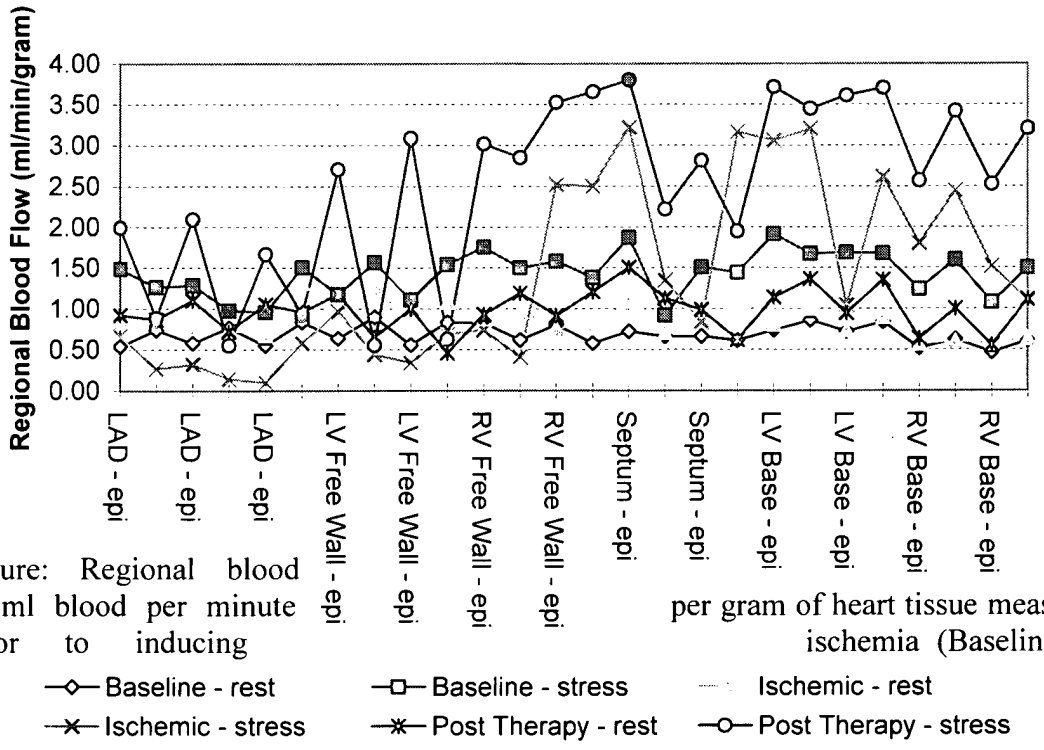

Figure: Regional blood flow in ml blood per minute per gram of heart tissue measured prior to inducing ischemia (Baseline), 3

weeks after placement of an ischemia-inducing ameroid constrictor (Ischemic), or 3 weeks after gene transfer into the ischemic heart (Post Therapy). At each time point, microspheres were injected during rest and stress conditions. Following the final tests, the pig was sacrificed and heart samples collected to determine regional blood flow.

Expression of vascular endothelial growth factor

pCI-VEGF was delivered to ischemic pig heart cells as described above. Plasma VEGF levels were measured in blood aspirated from the left atrial catheter using a commercially available ELISA assay (R&D Systems, Minneapolis, MN). Samples were immediately centrifuged for 10 minutes at 3,600 rpm and the serum stored at –20 _C until analysis. The assay is specific for the human VEGF protein, and does not measure pig VEGF. Two days following gene transfer of 7.5 mg pCI-VEGF, a level of 21.0 pg/ml human VEGF was detected. In 2 other pigs, levels of 19.6 and 15.2 pg/ml plasma were measured 3 days after administration of 2 mg pCI-VEGF.